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STUDIES ON COMPLEMENT FIXATION

V. THE HEMOLYTIC VERSUS FIXABILITY POWERS OF COMPLEMENT

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INTRODUCTION

There is perhaps no element in the complement-fixation phenomenon worthy of more careful investigation than the relation between the hemolytic and fixability properties of complement. The universal employment in fixability tests of measured amounts of complement based on hemolytic potency assumes not only a qualitative relation between these two properties but a quantitative relation as well. And yet it is becoming more and more emphasized that complement of good hemolytic titer may lack the power of being "fixed" by antigen-antibody complexes. The recent studies of Wilson,¹ Ruediger,² Kolmer and co-workers³ appear to lend fresh support to this view. Furthermore, according to the latter, the testing of the fixability of complement before its employment in the tests, as suggested by some workers, would appear to be of little value, as their results indicate that "a complement may be fixed by one syphilitic serum and not by another."

On the other hand, when one considers the numerous pitfalls associated with fixability tests, one is inclined to believe that the so-called nonfixability of some complement may not be due to the nature of the complement but rather to a combination of factors entering into the tests. To enumerate some of these pitfalls, we have, first, the amount of complement used. Thus a complement of good hemolytic potency used in 1:5, 1:10 or 1:20 dilutions, in nontitrated amounts, may frequently represent more than a given serum and antigen are capable of binding. And, if the amount of complement left unbound is large enough hemolysis would result and that serum would pass as negative. The titration of complement and its use, let us say, in 2 unit quantities in the tests, does not exclude the possibility of unintentionally using an excess of this ingredient, in some cases. Identical complement titrations

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¹ Jour. Immunol., 1918, 3, p. 346.

² Jour. Infect. Dis., 1919, 24, p. 120.

³ Jour. Syph., 1919, 3, p. 407. This paper gives a résumé of the literature.

carried out by 2 workers sometimes vary in their results to the extent of 25% or more. When one considers the liberal amount of hemolysin commonly employed after the fixation period, we can again see why occasionally some positive serums may give the appearance of being weak or even negative.

Furthermore, the mode of fixation plays an important rôle when testing the fixability powers of a given serum and antigen. One of us has shown⁴ that the rate of fixation of complement is directly proportional to the concentration of antibodies in the immune serum. A serum of high concentration will fix a considerable amount of complement immediately on mixing the ingredients, while one of low concentration will frequently fix no more than 50% of complement after an incubation period of one hour in the water bath. It is conceivable, therefore, when testing the fixability of complement with a number of syphilitic serums, that those which just approach + + + +, would be weakly positive or negative when a fixation period of one hour in the water bath is employed.

In so far as the complement fixation test in syphilis is concerned, the type of antigen employed also appears to play an important rôle when testing the fixing powers of some positive serums. When studying the potency of different Wassermann antigens, we have observed again and again that a Noguchi antigen will give a negative result with a syphilitic serum which is positive with alcoholic extract and cholesterolized antigens.

Finally, the false negative results frequently caused by the presence of natural hemolysin in human serum, and to a lesser degree in guinea-pig serum, might also incorrectly be interpreted as being due to the nonfixability of complement.

These considerations led us to investigate the relation between hemolysis and fixation of complement. More specifically, our aim was first to find some good hemolytic complement lacking fixability, and, second, to investigate the underlying cause for its nonfixability. These studies were limited to syphilitic serums and Wassermann antigens.

EXPERIMENTS

The tests were made with a sheep-cell-guinea-pig-complement system. Every effort was made in the fixability tests to overcome those factors which occasionally lead to false negative reactions. Complement was used in carefully titrated amounts and none was used which con-

⁴ Kahn, R. L.: Jour. Exper. Med., 1921, 34, p. 217.

tained considerable amounts of natural hemolysin. The latter was removed from every serum tested by the method proposed by one of us.⁵ Fixation was carried out for 4 hours at icebox temperature.⁶ Altogether, the hemolytic and fixability powers of 478 guinea-pig complements were tested. A large number (203) of the pigs were rebled from time to time and their hemolytic titer and fixability retested. The total number of separate guinea-pig serums tested was 275.

The plan was to obtain the hemolytic unit of individual complements used in the Wassermann division of these laboratories and subsequently determine their relative fixability powers in the presence of some known syphilitic serum and antigen. Thus, if on a given day pooled complement of 4 guinea-pigs was used for the regular Wassermann tests, each of these complements was titrated for its hemolytic potency as well as for its fixability power. The syphilitic serums were those previously found to be + + + + with pooled complement from 3 to 6 guinea-pigs.

Complement was obtained by bleeding large guinea-pigs under anesthesia directly from the heart and was used after permitting the serum to remain with the clot for about 15 hours in the icebox. A hemolytic unit was taken to be the smallest quantity of complement which, in the presence of 0.1 c c (2 units) of hemolysin, laked 0.1 c c of a 5% sheep cell suspension after 15 minutes incubation in the water bath.

This unit was obtained by making a 1:10 dilution of complement with salt solution and titrating it in the following proportions:

	Tube									
	1	2	3	4	5	6	7	8	9	10
Complement (c c, 1:10).....	0.1	0.09	0.08	0.07	0.06	0.05	0.04	0.03	0.02	0.01
Hemolysin (c c, 2 units)....	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Sheep cells (c c, 5%).....	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Salt sol. (drops).....	2	2	2	3	3	3	3	4	4	4

The unit was read in each case after 15 minutes' incubation in the water bath, and two units were used in the complement-fixation tests. For the sake of uniformity as well as simplicity in pipetting, the complement in each case was so diluted as to include 2 units in 0.1 c c. Thus, if the unit of complement was 0.04 c c, instead of using 0.08 c c of a 1:10 dilution in the tests, 0.1 c c of a 1:12.5 dilution was employed. The antigen was an alcoholic extract of beef hearts,

⁵ Kahn, R. L.: Jour. Lab. & Clin. Med., 1921, 6, p. 218.

⁶ Kahn, R. L.: Arch. Dermat. & Syph., 1921, 4, p. 358.

previously freed from ether soluble lipoids. Its mode of preparation is described in another paper.⁷ The tests were read in each case after permitting the tubes to remain in the icebox over night.

TABLE 1
RELATION BETWEEN HEMOLYTIC AND FIXABILITY POWERS OF COMPLEMENT

Guinea-Pig Complement			Syphilitic Serum, C c						
Laboratory No.	Titration Unit, C c	Dilution Representing 2 Units†	No.	Results of Fixability Tests					
				0.02	0.01	0.005	0.003	0.002	0.001
G-37	0.07	1:7	1	4*	4	3	1	1	—
F-24	0.04	1:12.5		4	4	4	1	—	—
B-19	0.08	1:6		4	4	1	—	—	—
E-3	0.05	1:10	2	4	4	4	4	4	1
F-15	0.06	1:8		4	4	4	3	1	1
E-23	0.05	1:10		4	4	4	4	4	1
E-21	0.05	1:10	3	4	4	4	4	4	1
B-4	0.04	1:12.5		4	4	4	4	4	1
E-16	0.05	1:10		4	4	4	4	1	—
E-20	0.04	1:12.5	4	4	4	4	4	4	1
E-17	0.04	1:12.5		4	4	4	4	3	1
E-19	0.04	1:12.5		4	4	4	4	3	1
J-5	0.04	1:12.5	5	4	4	4	4	3	1
J-25	0.04	1:12.5		4	4	4	4	2	1
E-11	0.03	1:16.5		4	4	4	4	3	1
E-12	0.03	1:16.5	6	4	4	4	4	4	2
G-39	0.03	1:16.5		4	4	4	4	4	4
E-22	0.03	1:16.5		4	4	4	4	4	4
E-2	0.04	1:12.5	7	4	4	4	4	4	4
B-13	0.04	1:12.5		4	4	4	4	4	1
A-50	0.03	1:16.5		4	4	4	4	4	4
A-23	0.03	1:16.5	8	4	4	4	4	2	—
B-3	0.03	1:16.5		4	4	4	4	2	1
A-38	0.03	1:16.5		4	4	4	4	2	—
E-8	0.04	1:12.5	9	4	4	4	3	1	—
E-4	0.03	1:16.5		4	4	4	3	1	—
E-7	0.05	1:10		4	4	4	3	1	—
G-39	0.04	1:12.5	10	4	4	4	4	2	1
G-38	0.04	1:12.5		4	4	4	4	3	1
G-46	0.04	1:12.5		4	4	4	4	2	1
G-22	0.07	1:7		4	4	4	4	3	1

* 4 = + + + +; 3 = + + +; 2 = + +; 1 = +; — = negative.

† These dilutions were determined accordingly: Titration unit : 0.05 :: (1:10) : (1:X). Thus if 0.04 is the titration unit, 0.04 : 0.05 :: 10 : X, X = 12.5.

Now, if it is true that the phenomena of hemolysis and fixation are closely related, one might expect 2 unit quantities of complement obtained from different guinea-pigs to possess the same fixability powers when used with the same serum. We found this to be largely but not entirely true. Table 1 gives an outline of the first 10 of 120 experiments. These 10 experiments represent 31 different comple-

⁷ Kahn, R. L., and Olin, R. M., Jr.: Jour. Infect. Dis., 29, p. 630.

ments and illustrate the variations in fixability of 2 units of complement. It did not seem wise to present all the experiments in tabular form on account of the large space required and particularly because, in a general way, these 10 are representative of the rest of the work.

The chief reason why 2 unit quantities of different complements show varying degrees of fixability when tested with one serum, is undoubtedly that our method for titrating complement is not sufficiently precise; in other words, we believe that the comparatively small fluctuations in fixability of these quantities of complement as indicated in table 1, are due to small variations in the hemolytic potency of so-called 2 units. We hope to test the validity of this belief by developing a method for more precise titrations of complements and quantitatively test their fixability with the same serum.

The experiments in table 1 were continued until Oct. 25. The total number of complements tested to that date was 187—without finding a single one which did not possess fixability properties. Beginning with Oct. 26, these 2 changes in these experiments were made: whenever a given complement possessed a high hemolytic titer, it was used both in 2 unit quantities as well as in 1:10 quantities. Also, the methods of fixation resorted to were both, 1 hour in the water bath as well as 4 hours in the icebox. It was desired to determine whether the nonfixability of some complements reported by different investigators might not be due to brief fixation periods as well as to the employment of average amounts instead of carefully titrated amounts of complement.

Table 2 illustrates to what extent excessive amounts of complement and water-bath fixation will reduce the fixing power of syphilitic serum and antigen. Strongly positive serums did not show this tendency. These serums, as is well known, are capable of absorbing large amounts of complement and are not extensively affected by the mode of fixation. Serums which were approaching + + + +, ranged between + + and negative in practically every case, when 1:10 complement was employed with water-bath fixation.

At the time when these experiments were carried out, the routine Wassermann tests in this laboratory were carried out in duplication employing an alcoholic extract antigen with 4 hours' fixation at icebox temperature and a cholesterized antigen with a half hour fixation at water-bath temperature. Frequently, syphilitic serums were found to be + + + + with the alcoholic antigen and + + + with the choles-

terinized antigen. These serums were considered as approaching ++++, and with highly potent complement used 1:10 instead of 2 units, combined with water-bath fixation, were found without exception to be weakly positive or negative.

TABLE 2

EFFECT OF EXCESSIVE AMOUNTS OF COMPLEMENT AND WATER-BATH FIXATION ON THE FIXABILITY POWERS OF FIVE DIFFERENT COMPLEMENTS WITH A FOUR PLUS SYPHILITIC SERUM

Complement Unit, No.	Titration Unit, C c	Dilutions of Complement Employed in Fixation Tests (Amount 0.1 Cc)	Mode of Fixation	Results of Fixability Tests, C c					
				0.02	0.01	0.005	0.003	0.002	0.001
1	0.035	1:14.5* 1:10	4 hours in icebox..... 1 hour in water-bath.	4† 2	4 1	4 —	1 —	1 —	— —
2	0.03	1:16.5* 1:10	4 hours in icebox..... 1 hour in water-bath.	4 2	4 1	4 —	1 —	1 —	— —
3	0.035	1:14.5* 1:10	4 hours in icebox..... 1 hour in water-bath.	4 2	4 1	4 —	1 —	1 —	— —
4	0.035	1:14.5* 1:10	4 hours in icebox..... 1 hour in water-bath.	4 1	4 1	2 —	1 —	— —	— —
5	0.03	1:16.5* 1:10	4 hours in icebox..... 1 hour in water-bath.	4 1	4 1	2 —	1 —	— —	— —

* These dilutions represent 2 units of complement.

† 4 = ++++; 3 = +++; 2 = ++; 1 = +, and — = negative.

SUMMARY AND CONCLUSIONS

It has become a matter of wide acceptance among complement-fixation workers that no relation exists between the hemolytic and fixability powers of complement; that a given complement may be capable of laking corpuscles in the presence of specific hemolysin and not be capable of being "fixed" by some specific antigen-antibody complexes. This study was made with a view of finding nonfixable complements and determining the underlying cause or causes for their nonfixability. The hemolytic tests were carried out with a sheep-cell-guinea-pig-complement system and the fixability tests, with an alcoholic extract antigen of beef-heart and syphilitic serums. In these studies no complement was used which contained moderate amounts of natural amboceptor. In the fixability tests, the complement was used in carefully titrated 2 unit quantities and fixation was carried out for 4 hours at icebox temperature.

Of 478 guinea-pig complements, representing 275 separate pigs, tested for fixability, not one was found which lacked this property.

On the other hand, it was found when using an average dilution of 1:10 of highly potent complement (instead of 2 units); a 1-hour fixation period in the water-bath (instead of 4 hours in the icebox) and a positive serum which just approaches $++$, that the fixation ranged from $++$ to negative in practically every case. In other words, our results indicate that so far as syphilitic serums and Wassermann antigens are concerned, the so-called nonfixability of some complements reported by some investigators may be apparent rather than real, and if all precautions are taken to overcome those factors in complement fixation which may lead to false negative results, that complements which possess hemolytic properties will also be found to be fixable.